AAFS, February 2023

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The Development of a Next Generation Sequencing Panel Targeting Cannabinoid Synthase Genes to Distinguish Between Marijuana and Hemp

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- The opinions, finding, conclusions, or recommendations expressed in this presentation are those of the authors.

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Differentiation of Marijuana and Hemp-Statement of Problems

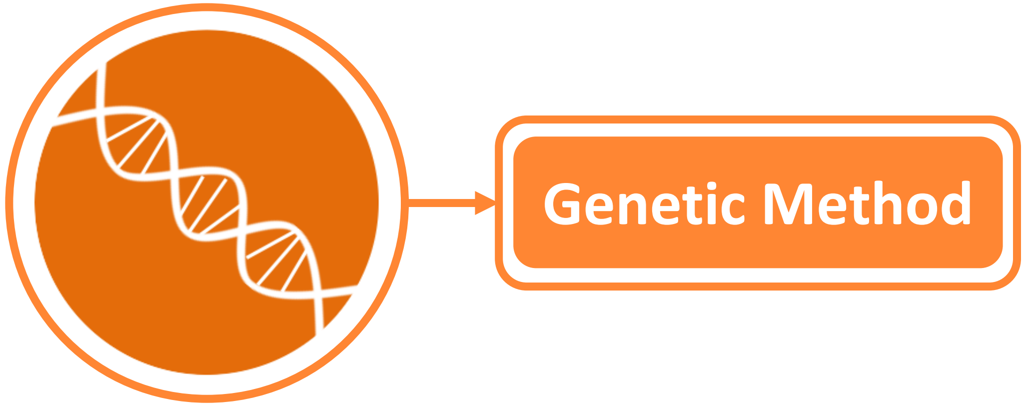
2018 Farm Bill

Trafficking

Method limitation

➢ Traditional Method Limitations

- Microscopic, Color test→ cannot differentiate hemp and marijuana
- Chemistry instrumentation→ provide limited information, effected by age, storage, sample type and size



Genetic Method

➢ Genetic Method

- Only need trace amount of sample available
- Analyzed non-traditional sample types
- Not effect by sample condition or storage
- Crop type differentiation
- Tracing geographic origin

Genetic Markers for Differentiating Hemp and Marijuana

Int J Legal Med (2016) 130:635–647

Legal Medicine 26 (2017) 33–40

LEGAL MEDICINE

plants

MDPI

Article

Developing and

(Hemp) for The

TECHNICAL NOTE

GENERAL

Cristiane Barbosa D'Oliveira Mati
M.S.; Lilian de Oliveira Machado,
Dobbler,[4] M.S.; Renata Machado
Roesch,[4] Ph.D.; Flávio Anastácio

Whole Plastome Seq
Drug-Type *Cannabis*: Insights into t
Use of Plastid in Forensic Analyses

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DNA polymorphisms in the tetrahydrocannabinolic acid (THCA) synthase gene in "drug-type" and "fiber-type" *Cannabis sativa* L.

Mareshige Kojoma[a,b,*], Hikaru Seki[b], Shigeo Yoshida[b], Toshiya Muranaka[b]

[a] *JYUGEI Institute (The Arboricultural Research Institute), University Forests, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 457 Kano, Minami-izu, Shizuoka 451-0304, Japan*
[b] *Plant Science Center, RIKEN, 1-7-22 Suehirocho, Tsurumi-ku, Yokohama 230-0045, Japan*

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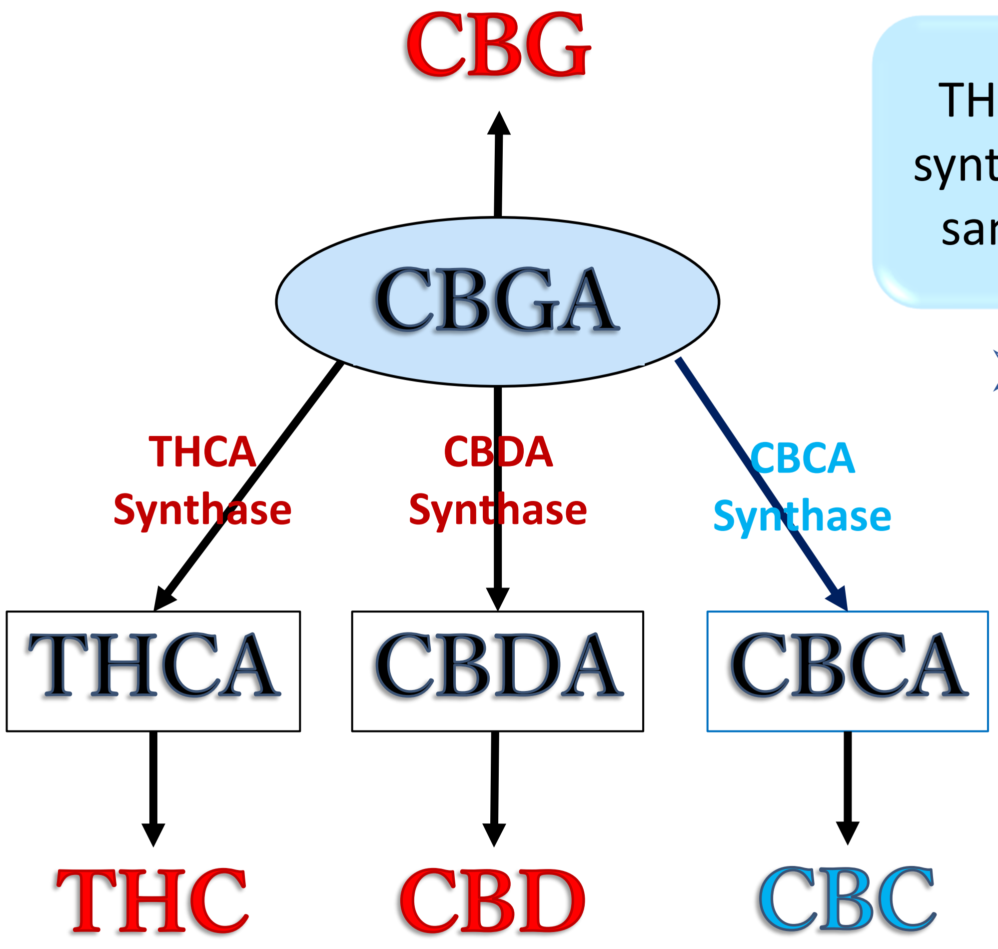
Synthase Gene and Chemotype

Cannabis strains are usually differentiated by their chemical composition (chemotype)



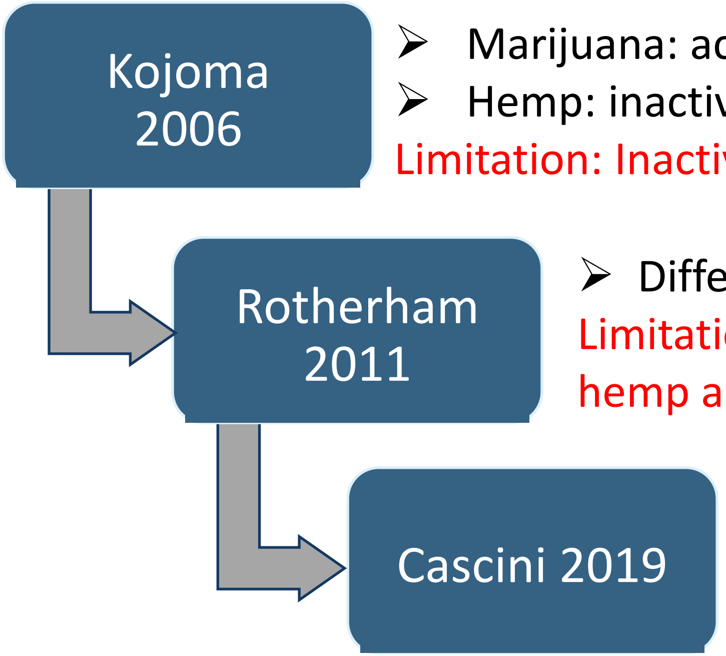
THCA, CBDA, and CBCA synthase compete for the same substrate (CBGA)

→ The genotypes differences of the three synthase genes might influence cannabinoid content → Differentiation Markers

➢ Synthase genes sequences

- CBCAS is reported to be 96% identical to THCAS sequence
- Cannabinoid synthase genes copies may include functional genes as well as several pseudogenes.
- Pseudogene with 89%– 95% similar in identity to THCAS, CBDAS, and CBCAS

Differentiating Hemp and Marijuana with Synthase Genes


Kojoma 2006

- ➢ Marijuana: active form of THCA synthase gene
- ➢ Hemp: inactive THCA synthase gene variant

Limitation: Inactive THCAS = CBCAS, present in both hemp and MJ (Laverty, 2019)

Rotherham 2011

- ➢ Differentiate Hemp and Marijuana using four SNPs on active and inactive gene

Limitation: False positive and false negative were found (Roman, 2022), mostly CBG hemp and seed samples.

Cascini 2019

- ➢ Investigate the relationship of chemotype with both THCAS and CBDAS gene
- ➢ The proposed fiber-type THCAS = CBCAS (Laverty, 2019)

Limitation: The risk of amplification of pseudogenes exists in all methods

➢ Limitation of Current Genetic method

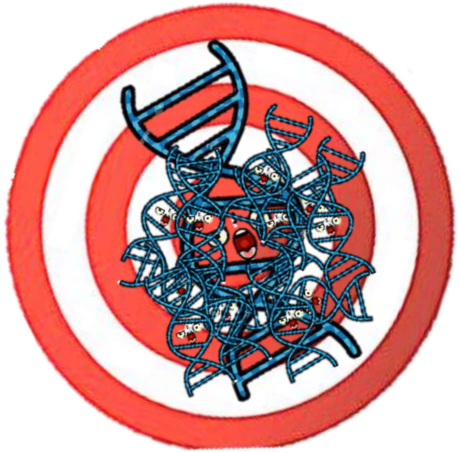
- **Need to incorporated CBCAS and pseudogene**
- **Limited sample varieties, small sample number (FP and FN still occured)**
- Complex genetic factors effecting potential markers
- Lack of a comprehensive *C. sativa* database

Study’s Goal and Approach

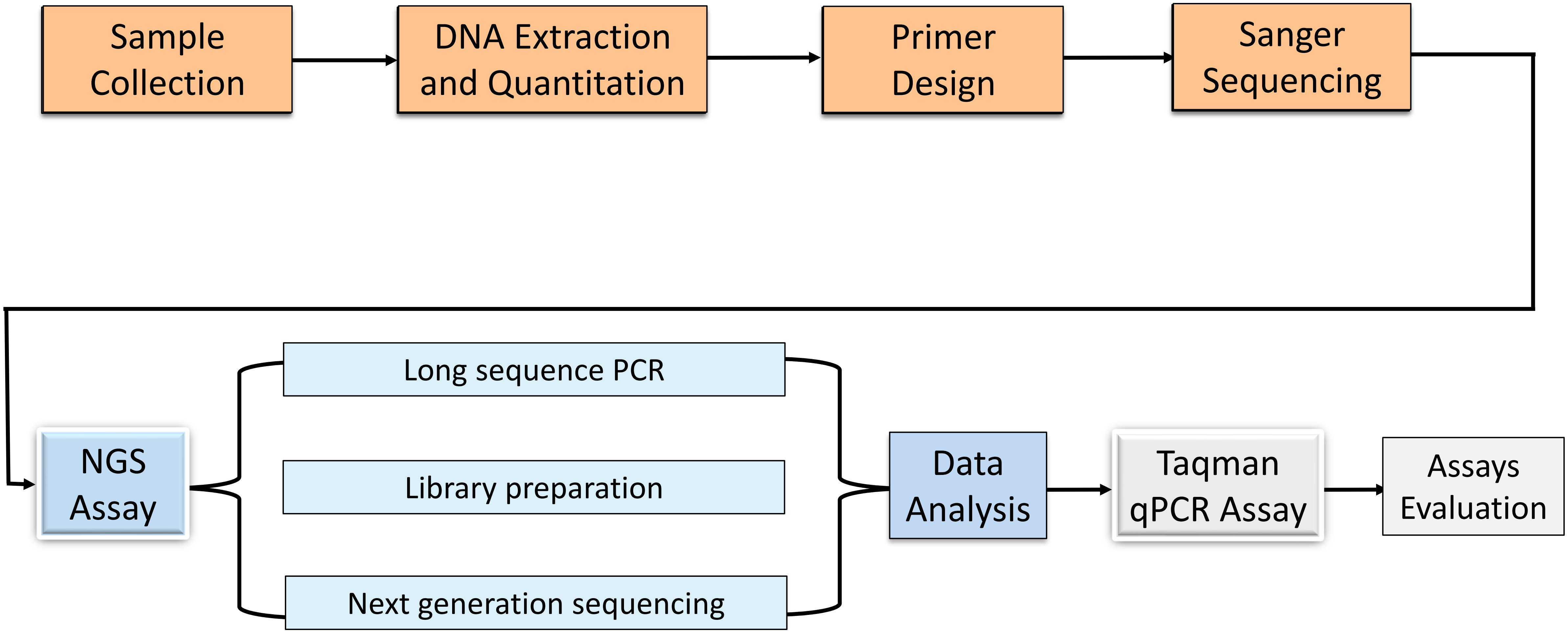
- **Goal**

Provide large scale evaluation of the synthase genes and pseudogenes on wide varieties of Cannabis to identify novel polymorphisms or pattern of genes’ present for crop type discrimination.

- **Designed NGS panel targeting three synthase genes and pseudogenes**
  - NGS can target specific genes of interest with high coverage and confidence
  - Quickly screen and sequence several types of cannabis with its high-throughput capabilities



Flow Chart



Sample Collection

DNA Extraction and Quantitation

Primer Design

Sanger Sequencing

NGS Assay

Long sequence PCR

Library preparation

Next generation sequencing

Data Analysis

Taqman qPCR Assay

Assays Evaluation

Cannabis Sample Collection

Crop Types		Number	Source	Chemistry Data
1	**Reference Marijuana**	27	NIDA, NIST	Quant & TIC Data
2	**Reference Hemp**	3	NIDA, NIST, University of Kentucky	Quant & TIC Data
3	**Seized Marijuana**	61	DEA (University of Mississippi)	Quant Data
4	**CBD Hemp**	20	Online purchase	Quant & TIC Data
5	**CBG Hemp**	13	University of Mississippi	Not Available
	CBG Hemp	8	Online purchase	Quant & TIC Data
6	**Δ8-THC Hemp**	7	Online purchase	Quant & TIC Data
7	**THCO Hemp**	3	Online purchase	Quant & TIC Data
8	**Δ10-THC Hemp**	1	Online purchase	Quant & TIC Data
9	**CBDV Hemp**	1	Online purchase	Quant & TIC Data
10	**Hemp Seed**	4	Grocery Store purchase	Not Available
	Total	**148**	**Marijuana: 88**	**Hemp: 60**

Primer Design

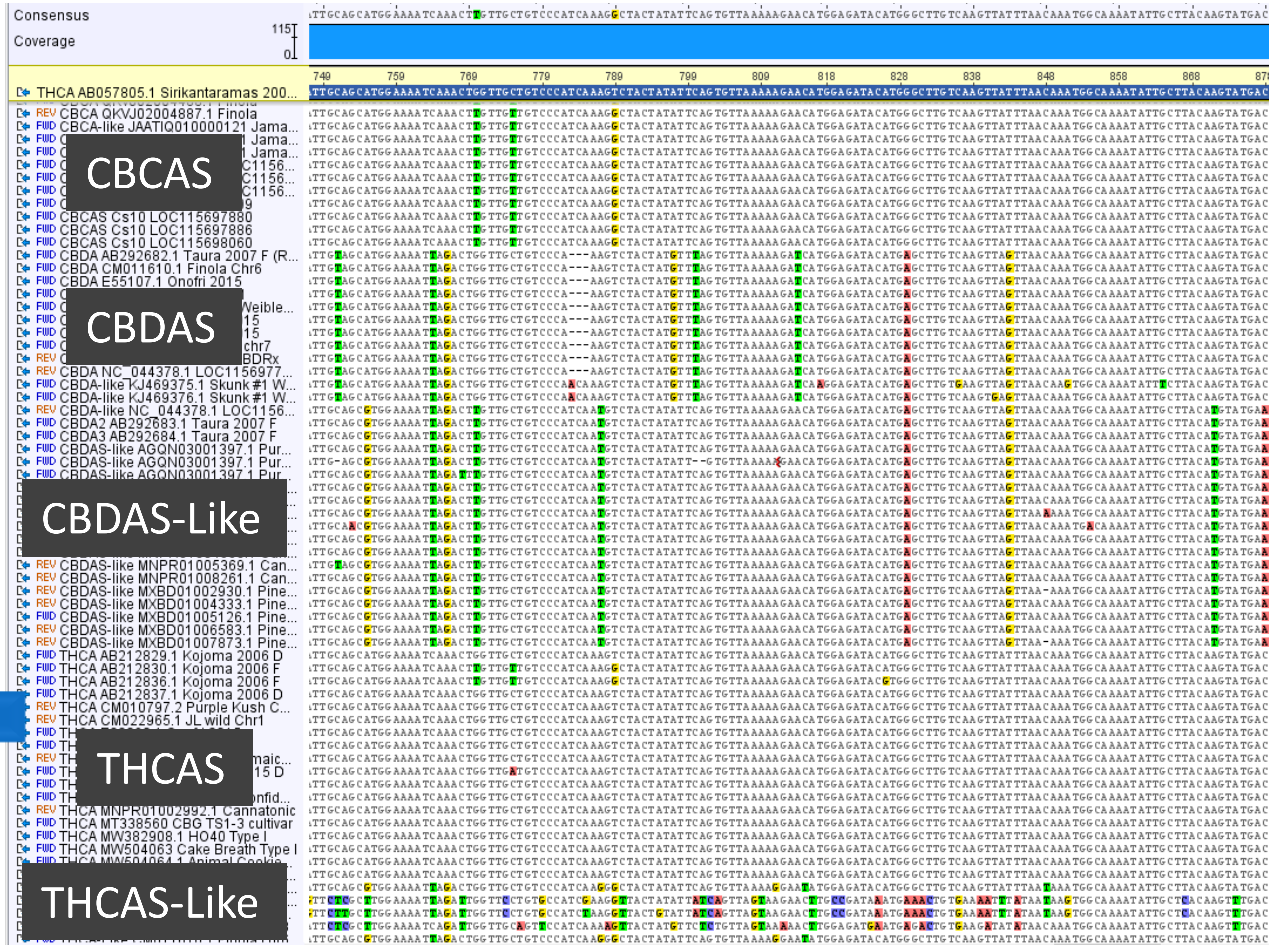
Approach 1: Using Published Primers

Name	Primer	Reference
THCAS F2	ACTGAAGAAAAATGAATTGCTCAG	**Cascini 2019**
THCAS R2	ATTTAAAGATAATTAATGATGATGCG	**Cascini 2019**
CBDAS F1	ATGAAGTGCTCAACATTCTC	**Cascini 2019**
CBDAS Lav F	CTGCAGGAATGAAGTACTCAACATTCTCCTTTTGG	**Laverty 2019**
CBDAS R1	TTAATGACGATGCCGTGG	**Cascini 2019**
CBDAS Lav R	AAGCTTTCATGGTACCCCATGATGATGCCGTGGAAGAG	**Laverty 2019**
CBCAS Lav F	CGGATGTACTGTTATGCTCCAA	**Laverty 2019**
CBCAS Lav R	CATTCTCCATTAAAATAAGAAAGACAA	**Laverty 2019**

No.	Primer Name	No.	Primer Name	No.	Primer Name
1	THCAS F4	8	CBCAS R2	15	CBDA+Like F2
2	THCAS R4	9	THCA+Like R	16	CBDA+Like F3
3	CBDAS F3	10	Everyone R1	17	CBCA+Like F
4	CBDAS R3	11	CBDA+Like R1	18	THCA F5
5	CBCAS F1	12	THCA R5	19	Everyone R2
6	CBCAS F3	13	CBDA+Like R2	20	Everyone F1
7	CBCAS R1	14	CBDA+Like F1		

Approach 2: Aligned Published Synthases' Sequence

Align 121 Reference Sequences (including THCAS, THCAS-like, CBDAS, CBDAS-like, CBCAS)

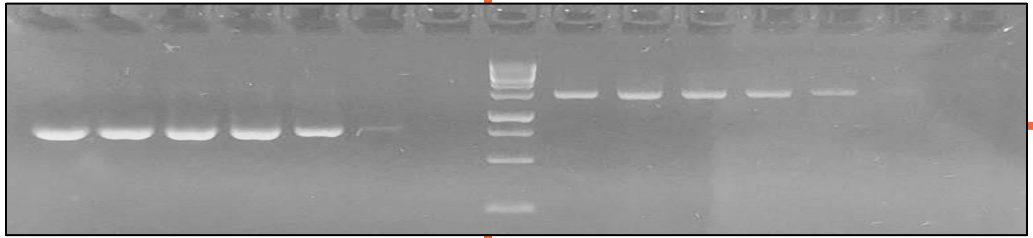


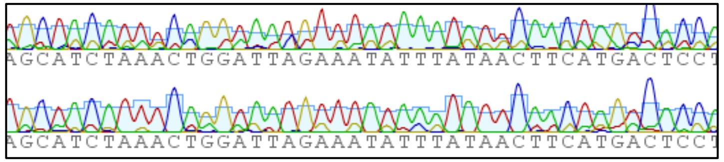
Primer Design-Pseudo Synthase Genes

Possible primers

Primer Name
THCA+Like R
Everyone R1
CBDA+Like R1
THCA R5
CBDA+Like R2
CBDA+Like F1
CBDA+Like F2
CBDA+Like F3
CBCA+Like F
THCA F5
Everyone R2
Everyone F1

Check different combinations

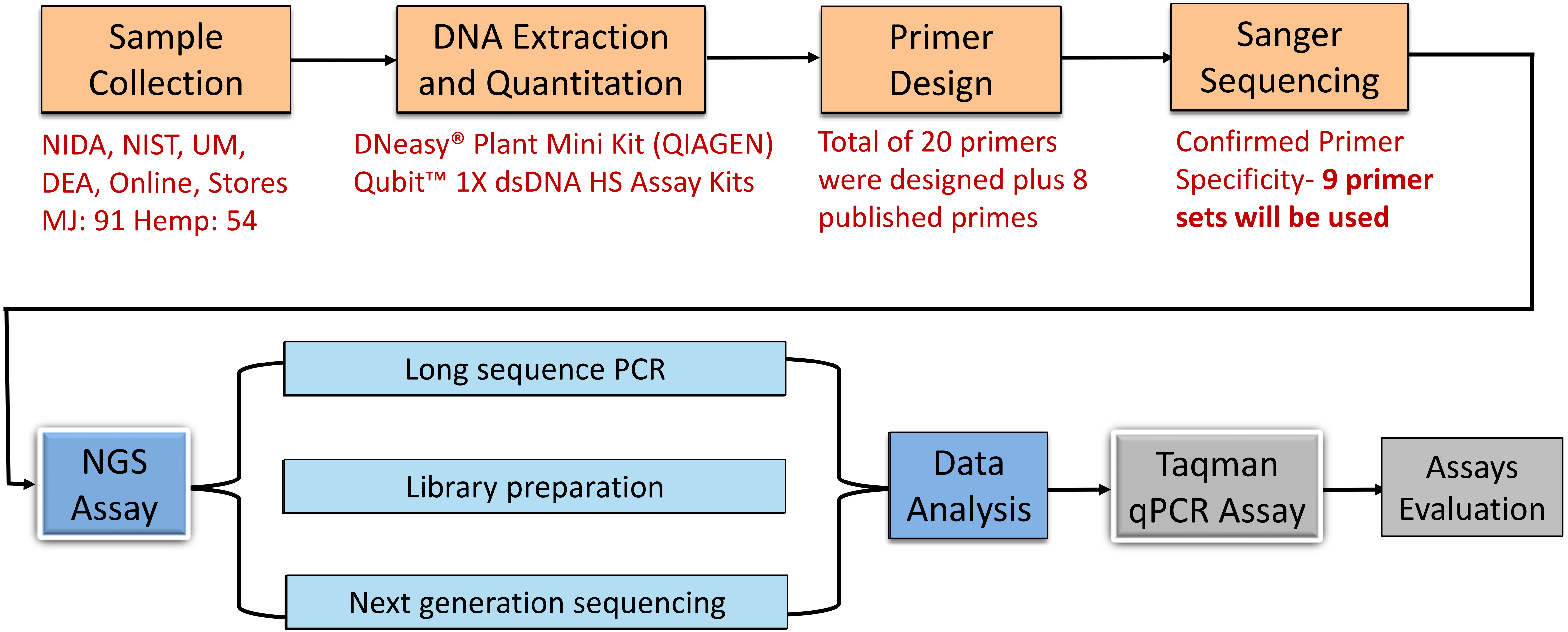




Larger cover range, good gel results, good specificity

Set	Name	Product	Target	Tm ºC
1	THCA+Like R & Everyone F1	1095 bp	THCAS+Like	61
2	CBDA+Like R1 & CBDA+Like F1	950 bp	CBDAS+Like	55
3	CBDA+Like R1 & CBDA+Like F2	1400 bp	CBDAS+Like	55
4	CBDA+Like R1 & CBDA+Like F3	1600 bp	CBDAS+Like	55
5	CBDA+Like R2 & CBDA+Like F2	560 bp	CBDAS+Like	x
6	CBDA+Like R2 & CBDA+Like F3	760 bp	CBDAS+Like	61
7	CBCA+Like F & Everyone R	1255 bp	CBCAS+Like	66
8	THCA R5 & THCA F5	1600 bp	THCAS	55

Flow Chart



Sample Collection

NIDA, NIST, UM,
DEA, Online, Stores
MJ: 91 Hemp: 54

DNA Extraction and Quantitation

DNeasy® Plant Mini Kit (QIAGEN)
Qubit™ 1X dsDNA HS Assay Kits

Primer Design

Total of 20 primers were designed plus 8 published primes

Sanger Sequencing

Confirmed Primer Specificity- **9 primer sets will be used**

NGS Assay

Long sequence PCR

Library preparation

Next generation sequencing

Data Analysis

Taqman qPCR Assay

Assays Evaluation

General Workflow

Long-Range PCR

- LA PCR Kit Ver.2.1
- Single-plex PCR

PCR Product Pool

- Qubit quantitation
- PCR Products Pool (0.2 ng/µL)
- Pool all targets from the sample
- ExoI clean-up

Library Preparation

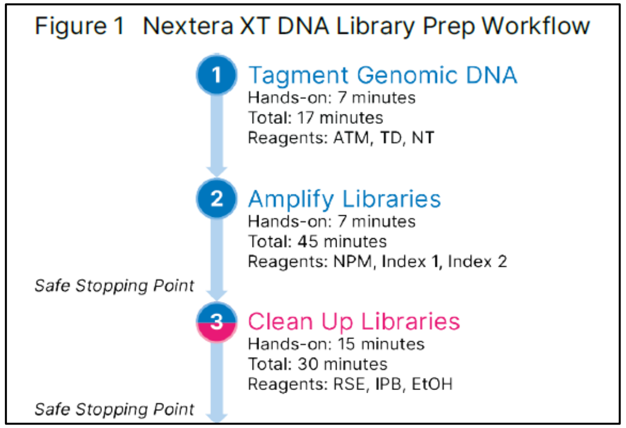


Figure 1 Nextera XT DNA Library Prep Workflow

1. Tagment Genomic DNA
   Hands-on: 7 minutes
   Total: 17 minutes
   Reagents: ATM, TD, NT
2. Amplify Libraries
   Hands-on: 7 minutes
   Total: 45 minutes
   Reagents: NPM, Index 1, Index 2

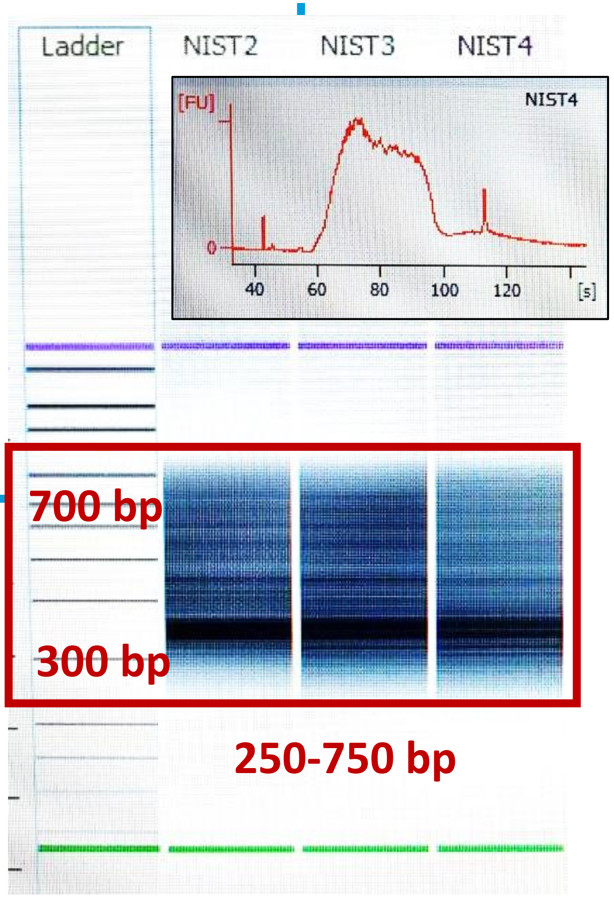
Safe Stopping Point

3. Clean Up Libraries
   Hands-on: 15 minutes
   Total: 30 minutes
   Reagents: RSE, IPB, EtOH

Safe Stopping Point

Libraries Quality Check

- Bioanalyzer



250-750 bp

MiSeq FGx Run

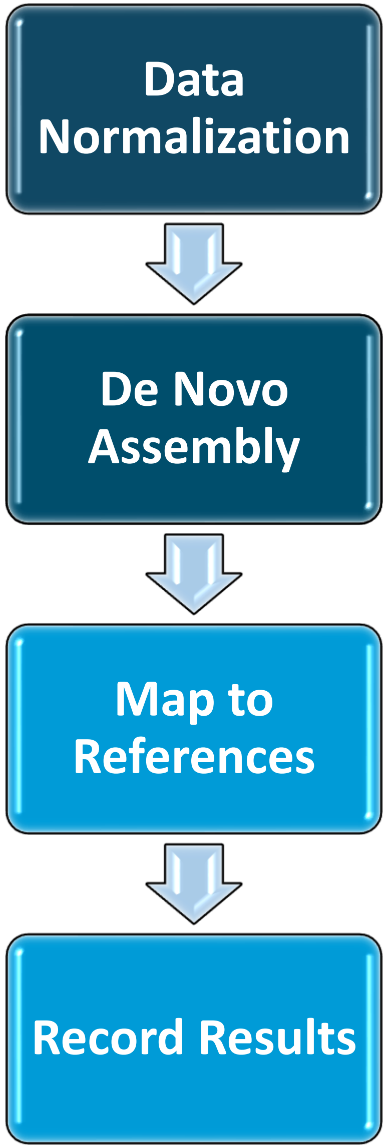
- Denature libraries
- Dilute denature libraries
- Instrument loading

Libraries Normalization

- Qubit quantitation
- Libraries Pool (1.32 ng/µL)

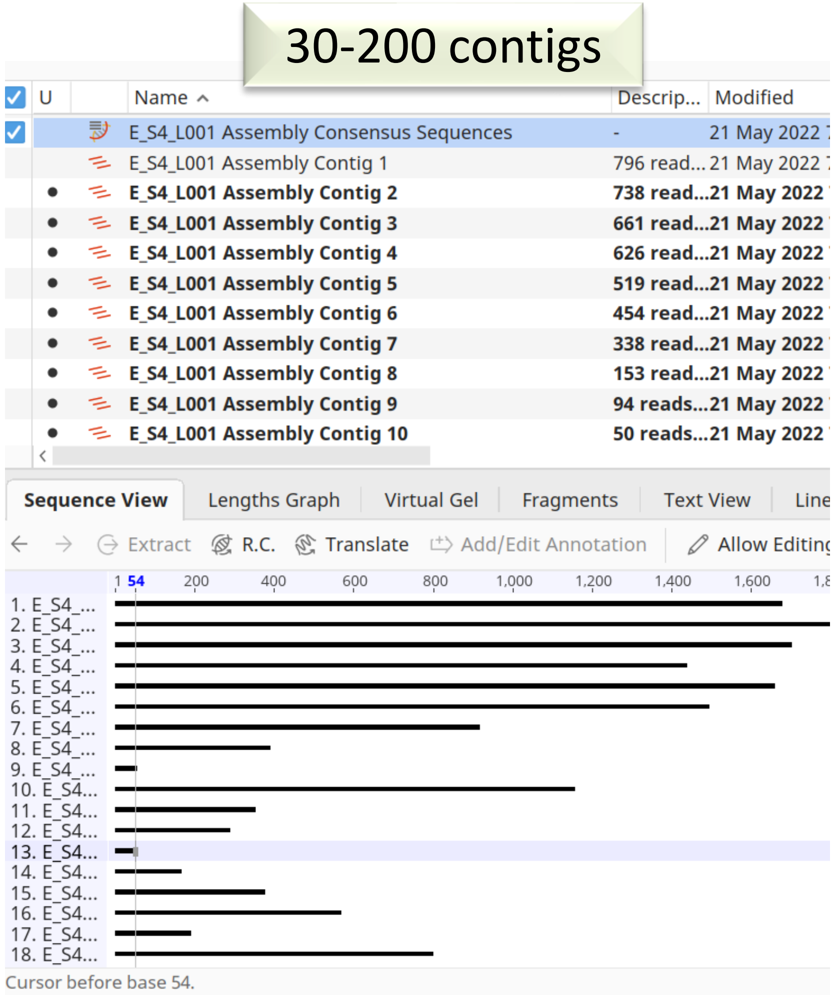
$$\text{Molarity} = \frac{\left(\frac{\text{ng}}{\mu\text{l}}\right)}{660\,\frac{\text{g}}{\text{mol}} \times 500\text{bp}}$$

Data Analysis-Geneious Prime



- Data Normalization
- De Novo Assembly
- Map to References
- Record Results

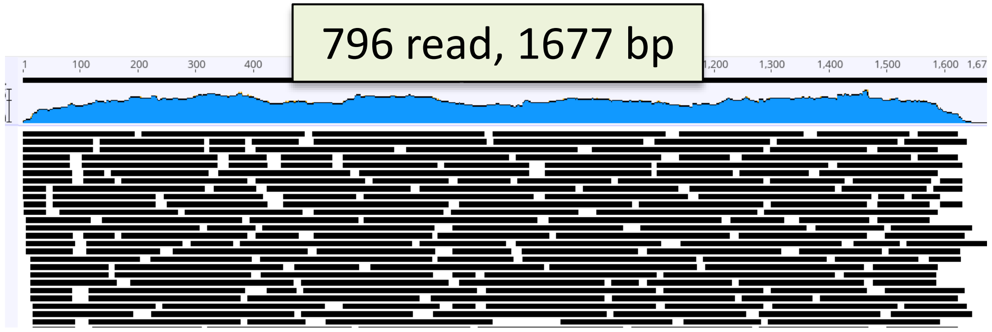
Assemble millions of reads generated by NGS platforms: align and merge overlapping fragments of a DNA sequence to reconstruct the original sequence.



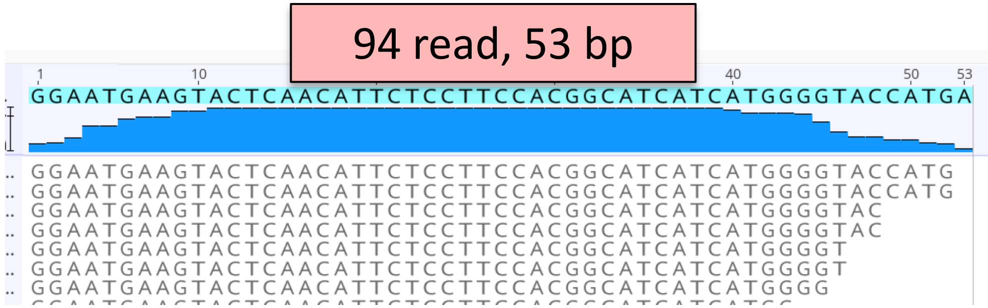
30-200 contigs

☑	U	Name ^	Descrip...	Modified
☑		E_S4_L001 Assembly Consensus Sequences	-	21 May 2022
		E_S4_L001 Assembly Contig 1	796 read...	21 May 2022
	●	**E_S4_L001 Assembly Contig 2**	**738 read...**	**21 May 2022**
	●	**E_S4_L001 Assembly Contig 3**	**661 read...**	**21 May 2022**
	●	**E_S4_L001 Assembly Contig 4**	**626 read...**	**21 May 2022**
	●	**E_S4_L001 Assembly Contig 5**	**519 read...**	**21 May 2022**
	●	**E_S4_L001 Assembly Contig 6**	**454 read...**	**21 May 2022**
	●	**E_S4_L001 Assembly Contig 7**	**338 read...**	**21 May 2022**
	●	**E_S4_L001 Assembly Contig 8**	**153 read...**	**21 May 2022**
	●	**E_S4_L001 Assembly Contig 9**	**94 reads...**	**21 May 2022**
	●	**E_S4_L001 Assembly Contig 10**	**50 reads...**	**21 May 2022**

Cursor before base 54.

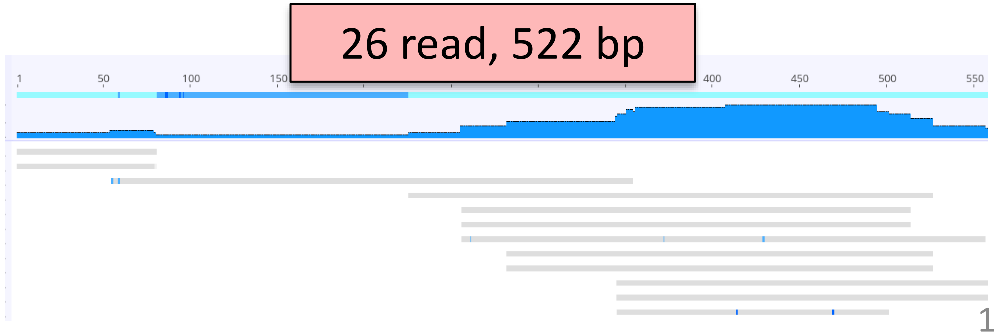


796 read, 1677 bp



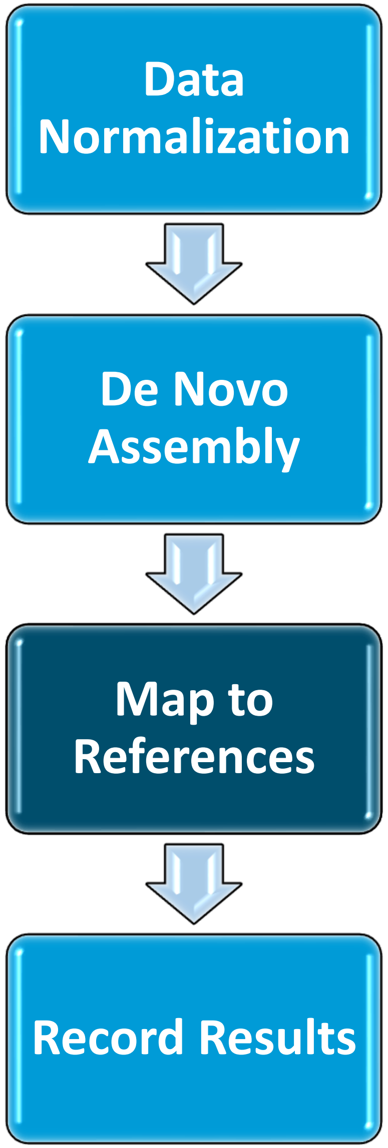
94 read, 53 bp

```
GGAATGAAGTACTCAACATTCTCCTTCCACGGCATCATCATGGGGTACCATGA
GGAATGAAGTACTCAACATTCTCCTTCCACGGCATCATCATGGGGTACCATG
GGAATGAAGTACTCAACATTCTCCTTCCACGGCATCATCATGGGGTACCATG
GGAATGAAGTACTCAACATTCTCCTTCCACGGCATCATCATGGGGTAC
GGAATGAAGTACTCAACATTCTCCTTCCACGGCATCATCATGGGGTAC
GGAATGAAGTACTCAACATTCTCCTTCCACGGCATCATCATGGGGT
GGAATGAAGTACTCAACATTCTCCTTCCACGGCATCATCATGGGGT
GGAATGAAGTACTCAACATTCTCCTTCCACGGCATCATCATGGGG
```



26 read, 522 bp

Data Analysis-Geneious Prime

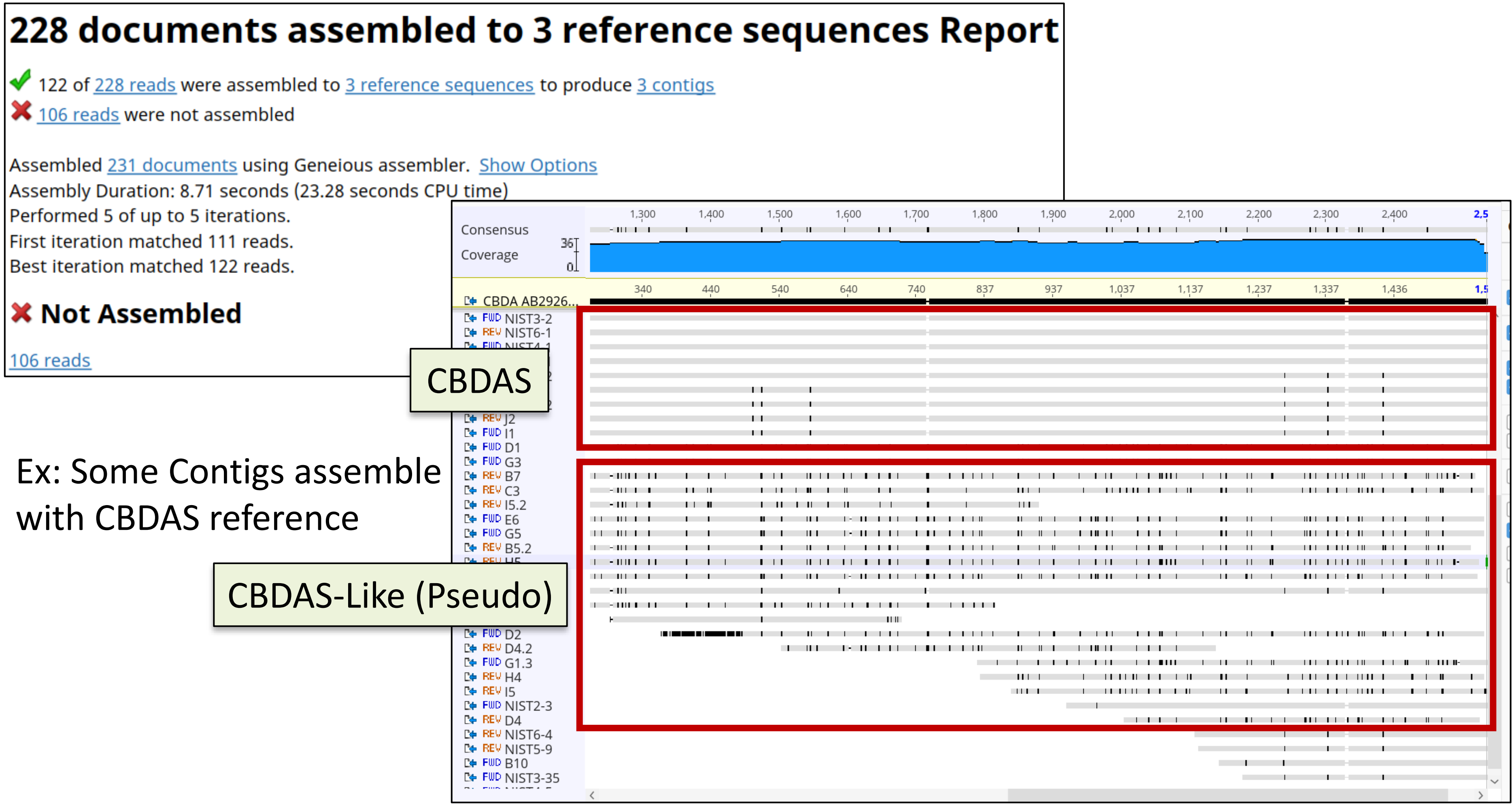

Data Normalization

De Novo Assembly

Map to References

Record Results

Map the contig to Reference Sequence: assemble contig sequences to THCAS, CBDAS, and CBCAS sequences to separate pseudo genes and locate differences


228 documents assembled to 3 reference sequences Report

✓ 122 of 228 reads were assembled to 3 reference sequences to produce 3 contigs

✗ 106 reads were not assembled

Assembled 231 documents using Geneious assembler. Show Options

Assembly Duration: 8.71 seconds (23.28 seconds CPU time)

Performed 5 of up to 5 iterations.

First iteration matched 111 reads.

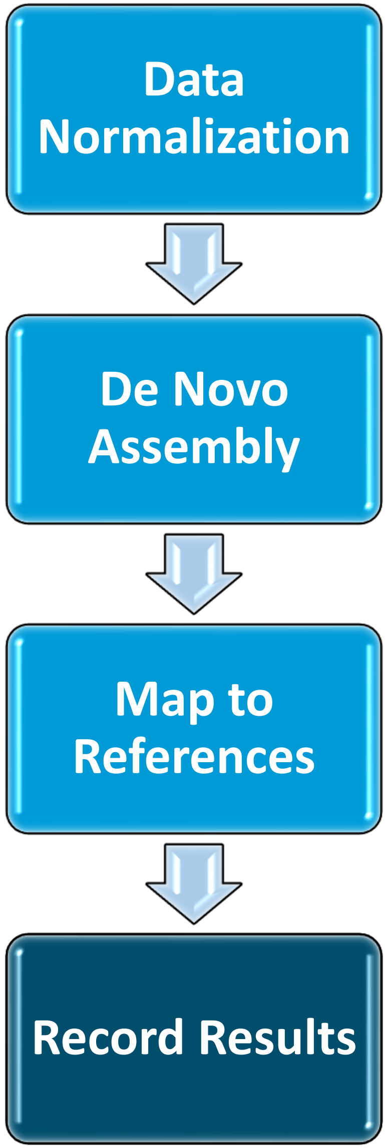
Best iteration matched 122 reads.

✗ Not Assembled

106 reads

Ex: Some Contigs assemble with CBDAS reference

Data Analysis-Geneious Prime

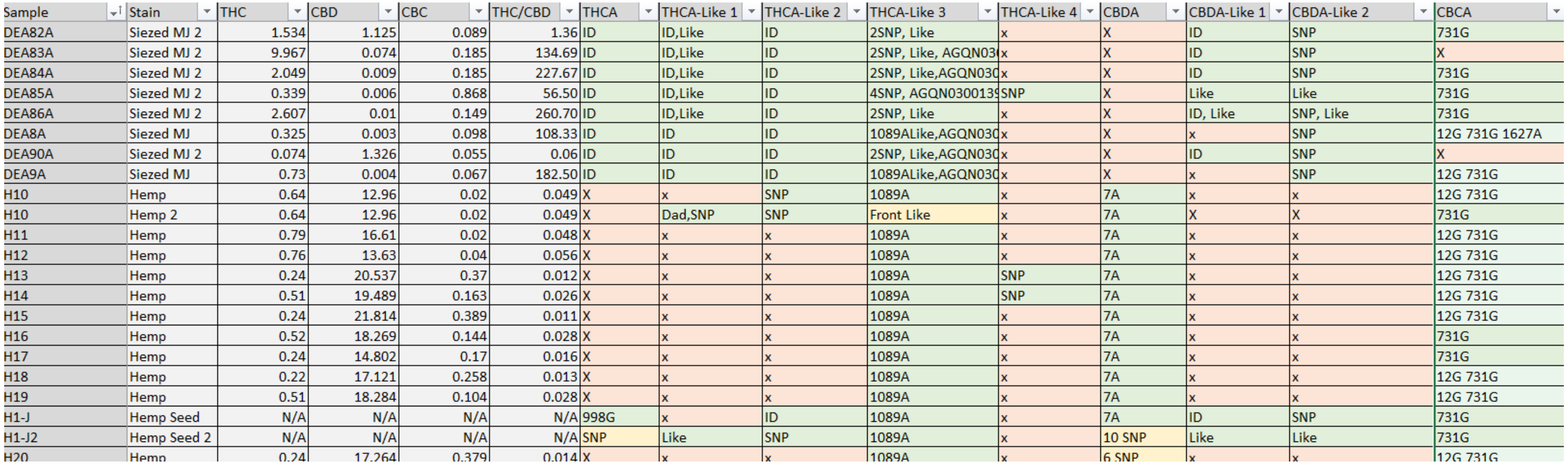

Data Normalization

De Novo Assembly

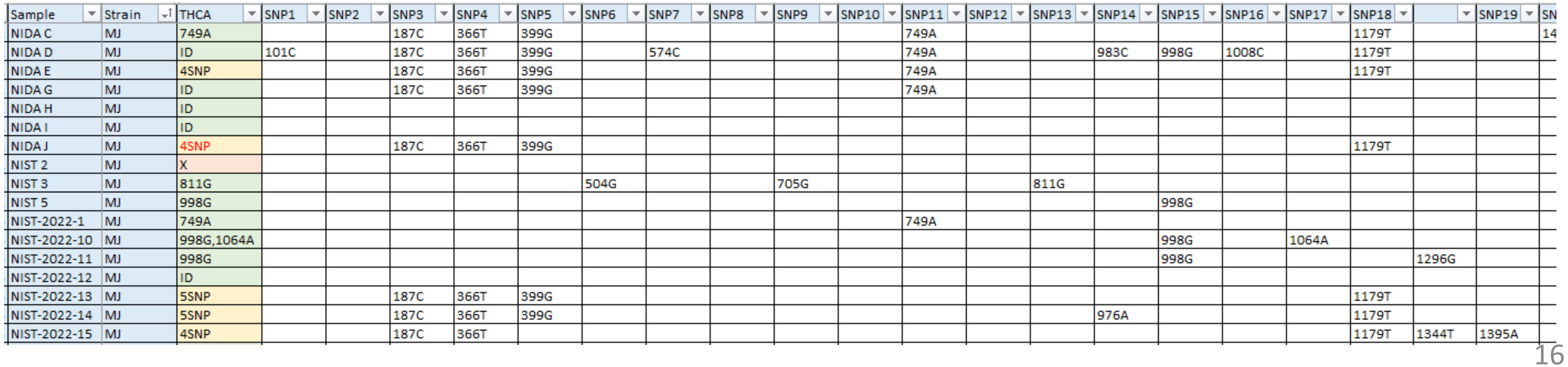
Map to References

Record Results

Excel Sheet: record present or absent of the synthase genes and pseudo synthase genes, as well as the polymorphisms on the synthase genes



Sample	Stain	THC	CBD	CBC	THC/CBD	THCA	THCA-Like 1	THCA-Like 2	THCA-Like 3	THCA-Like 4	CBDA	CBDA-Like 1	CBDA-Like 2	CBCA
DEA82A	Siezed MJ 2	1.534	1.125	0.089	1.36	ID	ID,Like	ID	2SNP, Like	x	X	ID	SNP	731G
DEA83A	Siezed MJ 2	9.967	0.074	0.185	134.69	ID	ID,Like	ID	2SNP, Like, AGQN03	x	X	ID	SNP	X
DEA84A	Siezed MJ 2	2.049	0.009	0.185	227.67	ID	ID,Like	ID	2SNP, Like,AGQN030	x	X	ID	SNP	731G
DEA85A	Siezed MJ 2	0.339	0.006	0.868	56.50	ID	ID,Like	ID	4SNP, AGQN030013	SNP	X	Like	Like	731G
DEA86A	Siezed MJ 2	2.607	0.01	0.149	260.70	ID	ID,Like	ID	2SNP, Like	x	X	ID, Like	SNP, Like	731G
DEA8A	Siezed MJ	0.325	0.003	0.098	108.33	ID	ID	ID	1089ALike,AGQN030	x	X	x	SNP	12G 731G 1627A
DEA90A	Siezed MJ 2	0.074	1.326	0.055	0.06	ID	ID	ID	2SNP, Like,AGQN030	x	X	ID	SNP	X
DEA9A	Siezed MJ	0.73	0.004	0.067	182.50	ID	ID	ID	1089ALike,AGQN030	x	X	x	SNP	12G 731G
H10	Hemp	0.64	12.96	0.02	0.049	X	x	SNP	1089A	x	7A	x	x	12G 731G
H10	Hemp 2	0.64	12.96	0.02	0.049	X	Dad,SNP	SNP	Front Like	x	7A	X	X	731G
H11	Hemp	0.79	16.61	0.02	0.048	X	x	x	1089A	x	7A	x	x	12G 731G
H12	Hemp	0.76	13.63	0.04	0.056	X	x	x	1089A	x	7A	x	x	12G 731G
H13	Hemp	0.24	20.537	0.37	0.012	X	x	x	1089A	SNP	7A	x	x	12G 731G
H14	Hemp	0.51	19.489	0.163	0.026	X	x	x	1089A	SNP	7A	x	x	12G 731G
H15	Hemp	0.24	21.814	0.389	0.011	X	x	x	1089A	x	7A	x	x	12G 731G
H16	Hemp	0.52	18.269	0.144	0.028	X	x	x	1089A	x	7A	x	x	731G
H17	Hemp	0.24	14.802	0.17	0.016	X	x	x	1089A	x	7A	x	x	731G
H18	Hemp	0.22	17.121	0.258	0.013	X	x	x	1089A	x	7A	x	x	12G 731G
H19	Hemp	0.51	18.284	0.104	0.028	X	x	x	1089A	x	7A	x	x	12G 731G
H1-J	Hemp Seed	N/A	N/A	N/A	N/A	998G	x	ID	1089A	x	7A	ID	SNP	731G
H1-J2	Hemp Seed 2	N/A	N/A	N/A	N/A	SNP	Like	SNP	1089A	x	10 SNP	Like	Like	731G
H20	Hemp	0.24	17.264	0.379	0.014	X	x	x	1089A	x	6 SNP	x	x	12G 731G



Sample	Strain	THCA	SNP1	SNP2	SNP3	SNP4	SNP5	SNP6	SNP7	SNP8	SNP9	SNP10	SNP11	SNP12	SNP13	SNP14	SNP15	SNP16	SNP17	SNP18		SNP19	SN
NIDA C	MJ	749A			187C	366T	399G						749A							1179T			14
NIDA D	MJ	ID	101C		187C	366T	399G		574C				749A			983C	998G	1008C		1179T			
NIDA E	MJ	4SNP			187C	366T	399G						749A							1179T			
NIDA G	MJ	ID			187C	366T	399G						749A										
NIDA H	MJ	ID																					
NIDA I	MJ	ID																					
NIDA J	MJ	4SNP			187C	366T	399G													1179T			
NIST 2	MJ	X																					
NIST 3	MJ	811G						504G			705G				811G								
NIST 5	MJ	998G															998G						
NIST-2022-1	MJ	749A											749A										
NIST-2022-10	MJ	998G,1064A															998G		1064A				
NIST-2022-11	MJ	998G															998G				1296G		
NIST-2022-12	MJ	ID																					
NIST-2022-13	MJ	5SNP			187C	366T	399G													1179T			
NIST-2022-14	MJ	5SNP			187C	366T	399G									976A				1179T			
NIST-2022-15	MJ	4SNP			187C	366T														1179T	1344T	1395A	

Results of Reference Cannabis Samples

Sample	Strain	THC/CBD	THCAS	CBDAS	CBCAS	THCAS-Like	CBDAS-Like	CBCAS-Like
NIST2022-7	Ref MJ	**0.027**	X	Present	Present	X or Present	X	X or Fragments
NIST2022-9	Ref MJ	**0.034**	X	SNPs	X	X or Present	X	X or Fragments
NIDA F	Ref Hemp	**0.036**	X	SNPs	Present	Present	X	X or Fragments
NIST2022-5	Ref MJ	**0.037**	X	Present	Present	Present	X	X or Fragments
NIST2022-4	Ref MJ	**0.038**	X	Present	Present	Present	X	X or Fragments
NIDA J	Ref MJ	**0.040**	X	SNPs	Present	Present	X	X or Fragments
NIST2022-3	Ref MJ	**0.044**	X	Present	Present	Present	X	X or Fragments
NIST2022-6	Ref Hemp	**0.044**	X	Present	Present	Present	X	X or Fragments
NIST2022-18	Ref MJ	**0.055**	Present	Present	Present	Present	Present	X or Fragments
NIST2022-17	Ref MJ	**0.073**	SNPs	Present	Present	Present	Present	X or Fragments
NIST2022-1	Ref MJ	**0.109**	SNPs	Present	Present	Present	Present	X or Fragments
NIST2022-11	Ref MJ	**0.242**	SNPs	Present	Present	Present	Present	X or Fragments
NIDA I	Ref MJ	**0.632**	Present	SNPs	Present	Present	Present	X or Fragments
NIDA H	Ref MJ	**0.649**	Present	SNPs	X	Present	Present	X or Fragments
NIST2022-15	Ref MJ	**1.129**	Present	Present	Present	Present	Present	X or Fragments
NIST2022-13	Ref MJ	**1.521**	SNPs	Present	Present	Present	Present	X or Fragments
NIST2022-10	Ref MJ	**1.546**	Present	Present	Present	Present	Present	X or Fragments
NIDA B	Ref MJ	**11.176**	Present	Present	Present	Present	Present	X or Fragments
NIST2022-14	Ref MJ	**11.419**	SNPs	Present	Present	Present	Present	X or Fragments
NIDA D	Ref MJ	**88.889**	Present	X	Present	Present	Present	X or Fragments
NIST2022-19	Ref MJ	**123.880**	Present	X	Present	Present	Present	X or Fragments
NIST2022-8	Ref MJ	**166.384**	SNPs	X	X	Present	Present	X or Fragments
NIST2022-20	Ref MJ	**240.895**	Present	X	Present	Present	Present	X or Fragments
NIDA G	Ref MJ	**252.500**	Present	X	Present	Present	Present	X or Fragments
NIDA E	Ref MJ	**335.000**	Present	X	Present	Present	Present	X or Fragments
NIDA C	Ref MJ	**390.000**	SNPs	X	Present	Present	Present	X or Fragments

Present or absent of THCAS, CBDAS and their pseudo genes are corresponding to THC/CBD ratio.

Results of Non-Reference Cannabis Samples

Sample	Strain	THCAS	CBDAS	CBCAS	THCAS-Like	CBDAS-Like
H23	**CBG Hemp**	SNP 1064G	X	X	Present	Present
CBG30A		SNP 1064G	Incomplete	Present	Present	Present
H22		SNP 1064G	X	X	Present	Present
H21		SNP 1064G	X	X	Present	Present
H41		SNP 1064G	X	X	Present	Present
H42		SNP 1064G	X	X	Present	Present
CBG59A		SNP 1064G	X	Incomplete	Present	Present
H26		SNP 1064G	X	X	Present	Present
CBG22A		SNP 1064G	X	X	Present	Present
CBG40A		SNP 1064G	Incomplete	X	Present	Present
CBG44A		SNP 1064G	Incomplete	X	Present	Present
CBG5A		SNP 1064G	X	X	Present	Present
CBG20B		SNP 1064G	Present	Present	Present	Present
CBG22B		SNP 1064G	X	Incomplete	Present	Present
CBG5B		SNP 1064G	X	Incomplete	Present	Present
H43		SNP 1064G	Incomplete	Incomplete	Present	Present

Sample	Strain	THCAS	CBDAS	CBCAS	THCAS-Like	CBDAS-Like
H15	**CBD Hemp**	X	Present	Present	X or Present	X
H13		X	Present	Present	X or Present	X
H18		X	Present	Present	X or Present	X
H20		X	SNPs	Present	X or Present	X
H24		X	Present	Present	X or Present	X
H14		X	Present	Present	X or Present	X
H19		X	Present	Present	X or Present	X
H11		X	Present	Present	X or Present	X
H10		X	Present	Present	X or Present	X
H9		X	Present	Present	X or Present	X
H12		X	Present	Present	X or Present	X
H47		X	Present	Present	X or Present	X
H45		X	Present	Present	X or Present	X
H44		X	Present	Present	X or Present	X
H46		X	Present	SNPs	X or Present	X
H25		X	Present	Present	X or Present	X

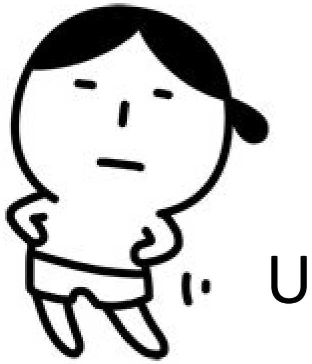
Sample	Strain	THCAS	CBDAS	CBCAS	THCAS-Like	CBDAS-Like
DEA27A	**Seized MJ**	Present	X	Present	Present	Present
DEA5A		Present	X	Present	Present	Present
DEA36A		Present	X	Present	Present	Present
DEA31A		Present	X	Present	Present	Present
DEA44A		Present	X	Present	Present	Present
DEA42A		Present	X	Present	Present	Present
DEA32A		Present	X	Present	Present	Present
DEA25A		Present	X	Present	Present	Present
DEA60A		Present	X	Present	Present	Present
DEA58A		SNPs	X	Present	Present	Present

Present or absent of THCAS, CBDAS and their pseudo genes are pretty consistent within CBG Hemp, CBD Hemp, and Seized MJ.

Non-Reference Cannabis Samples

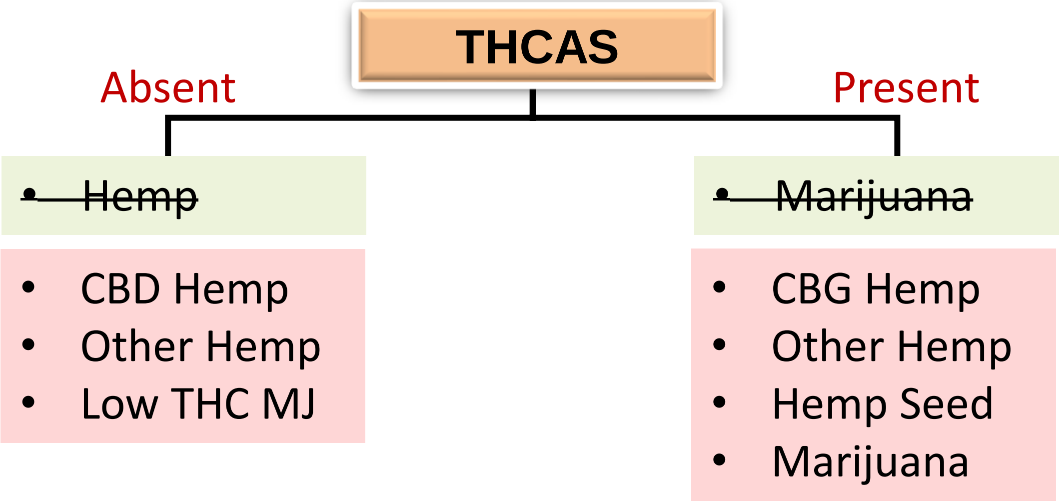
Sample	Strain	THCAS	CBDAS	CBCAS	THCAS-Like	CBDAS-Like
H39	Hemp CBDV	X	Present	Present	X	X
H1-J2	Hemp Seed 2	SNPs	SNPs	Present	SNPs	Present
H2-5	Hemp Seed 2	SNPs	SNPs	Present	SNPs	Present
H3-5	Hemp Seed 2	SNPs	SNPs	Present	SNPs	Present
H4-4	Hemp Seed 2	SNPs	SNPs	Present	SNPs	Present
H36	Hemp THCO	SNPs	Present	Present	Present	Present
H37	Hemp THCO	X	Present	Present	X	X
H35	Hemp THCO	X	Present	Present	X	X
H38	HempΔ10	SNPs	Present	Present	X	X
H28	HempΔ8	SNPs	Present	Present	SNPs	X
H29	HempΔ8	SNPs	Present	Present	SNPs	X
H31	HempΔ8	SNPs	Present	Present	SNPs	X
H30	HempΔ8	SNPs	Present	Present	X	Present
H34	HempΔ8	SNPs	Present	Present	Present	Present
H34	HempΔ8	X	Present	Present	X	Present
H32	HempΔ8	X	Present	Present	X	Present
H27	HempΔ8	X	Present	Present	X	X

Present or absent of THCAS, CBDAS and their pseudo genes are not really consistent between different hemp.



Umm…

Differentiate Hemp and Marijuana



THCAS

Absent

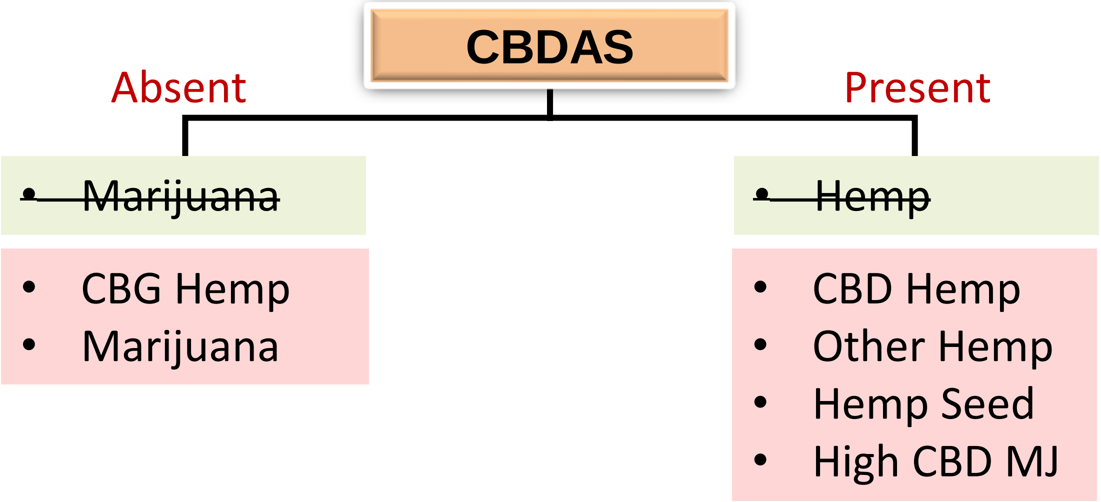
- ~~Hemp~~

- CBD Hemp
- Other Hemp
- Low THC MJ

Present

- ~~Marijuana~~

- CBG Hemp
- Other Hemp
- Hemp Seed
- Marijuana



CBDAS

Absent

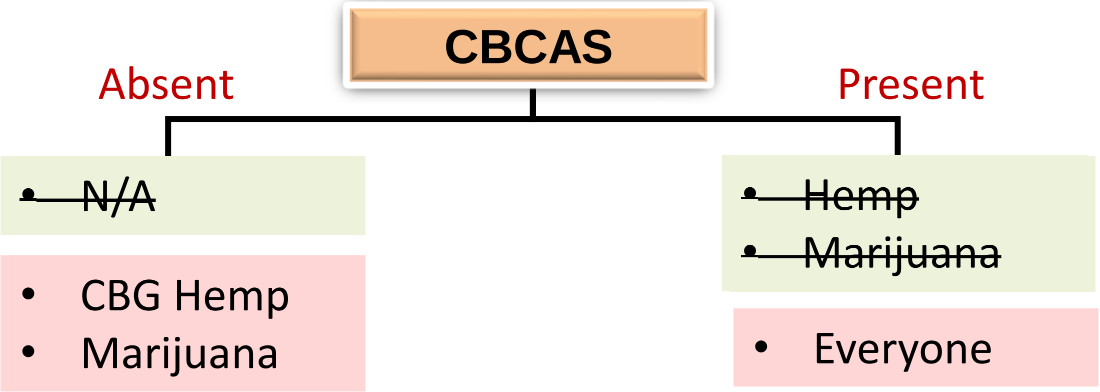
- ~~Marijuana~~

- CBG Hemp
- Marijuana

Present

- ~~Hemp~~

- CBD Hemp
- Other Hemp
- Hemp Seed
- High CBD MJ



CBCAS

Absent

- ~~N/A~~

- CBG Hemp
- Marijuana

Present

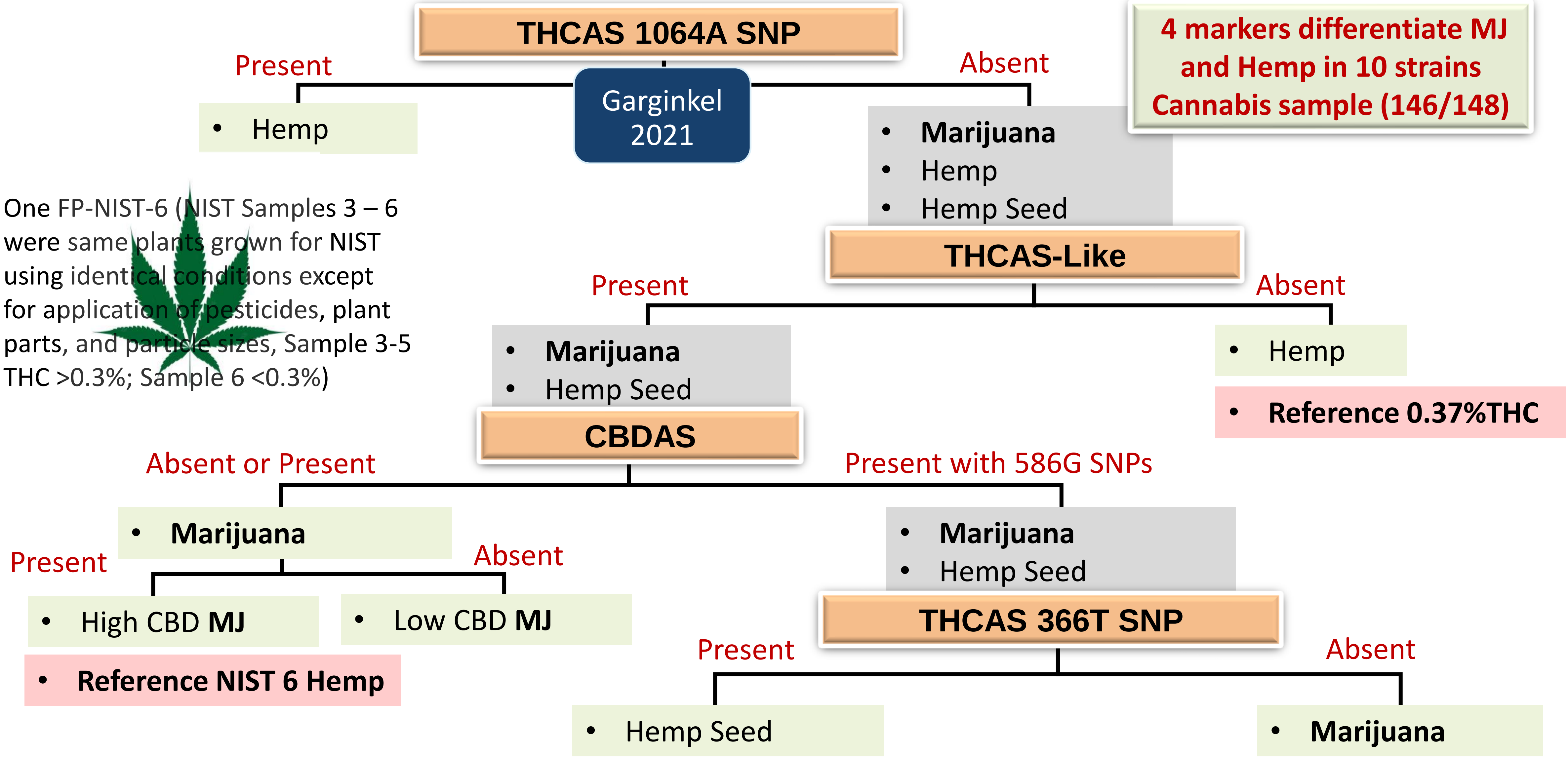
- ~~Hemp~~
- ~~Marijuana~~

- Everyone

Cannot differentiate hemp and marijuana by just synthase genes.

Cannot differentiate hemp and marijuana by looking at several genes "at the same time".

Differentiation Flow Chart



THCAS 1064A SNP

Garginkel 2021

Present
- Hemp

Absent
- **Marijuana**
- Hemp
- Hemp Seed

4 markers differentiate MJ and Hemp in 10 strains Cannabis sample (146/148)

One FP-NIST-6 (NIST Samples 3 – 6 were same plants grown for NIST using identical conditions except for application of pesticides, plant parts, and particle sizes, Sample 3-5 THC >0.3%; Sample 6 <0.3%)

THCAS-Like

Present
- **Marijuana**
- Hemp Seed

Absent
- Hemp
- **Reference 0.37%THC**

CBDAS

Absent or Present
- **Marijuana**

Present
- High CBD **MJ**
- **Reference NIST 6 Hemp**

Absent
- Low CBD **MJ**

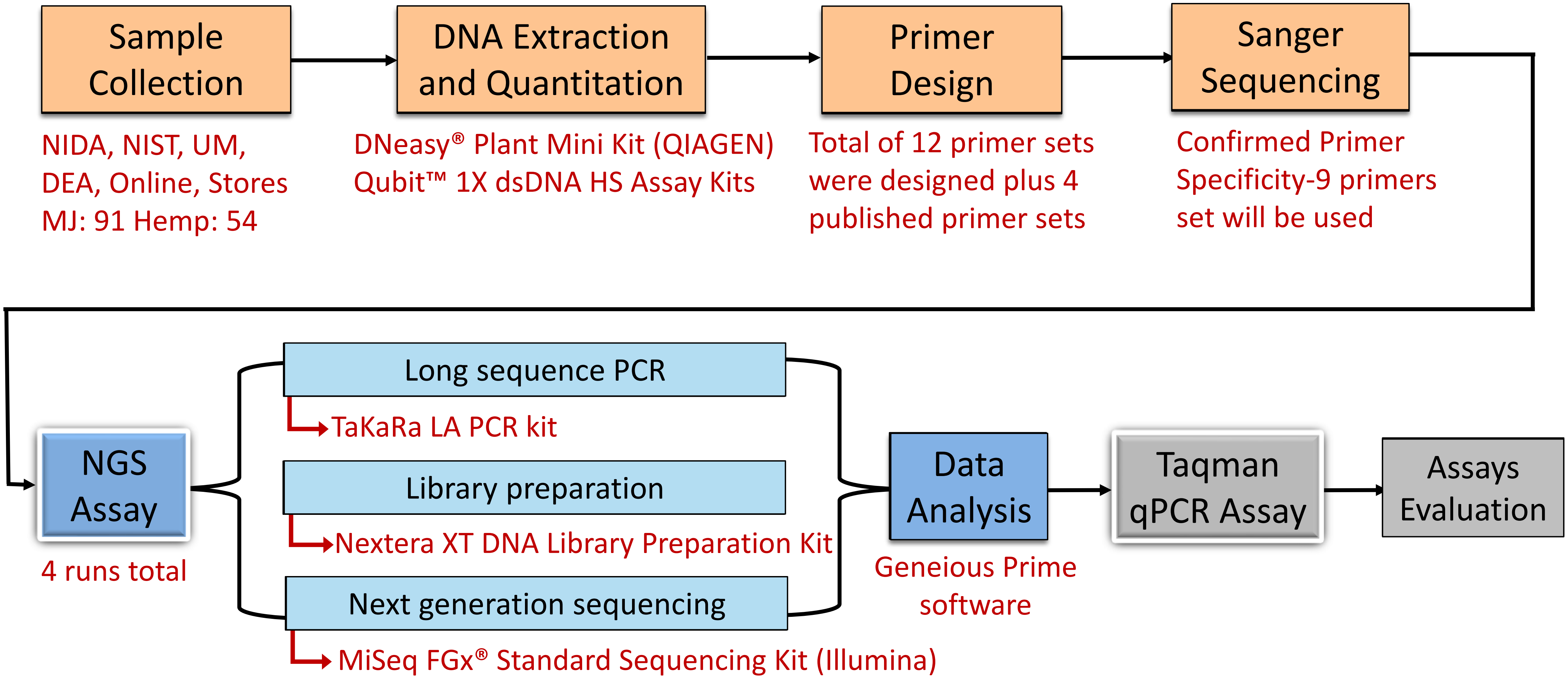
Present with 586G SNPs
- **Marijuana**
- Hemp Seed

THCAS 366T SNP

Present
- Hemp Seed

Absent
- **Marijuana**

Flow Chart- Next Step



Sample Collection

NIDA, NIST, UM,
DEA, Online, Stores
MJ: 91 Hemp: 54

DNA Extraction and Quantitation

DNeasy® Plant Mini Kit (QIAGEN)
Qubit™ 1X dsDNA HS Assay Kits

Primer Design

Total of 12 primer sets were designed plus 4 published primer sets

Sanger Sequencing

Confirmed Primer Specificity-9 primers set will be used

NGS Assay

4 runs total

Long sequence PCR

TaKaRa LA PCR kit

Library preparation

Nextera XT DNA Library Preparation Kit

Next generation sequencing

MiSeq FGx® Standard Sequencing Kit (Illumina)

Data Analysis

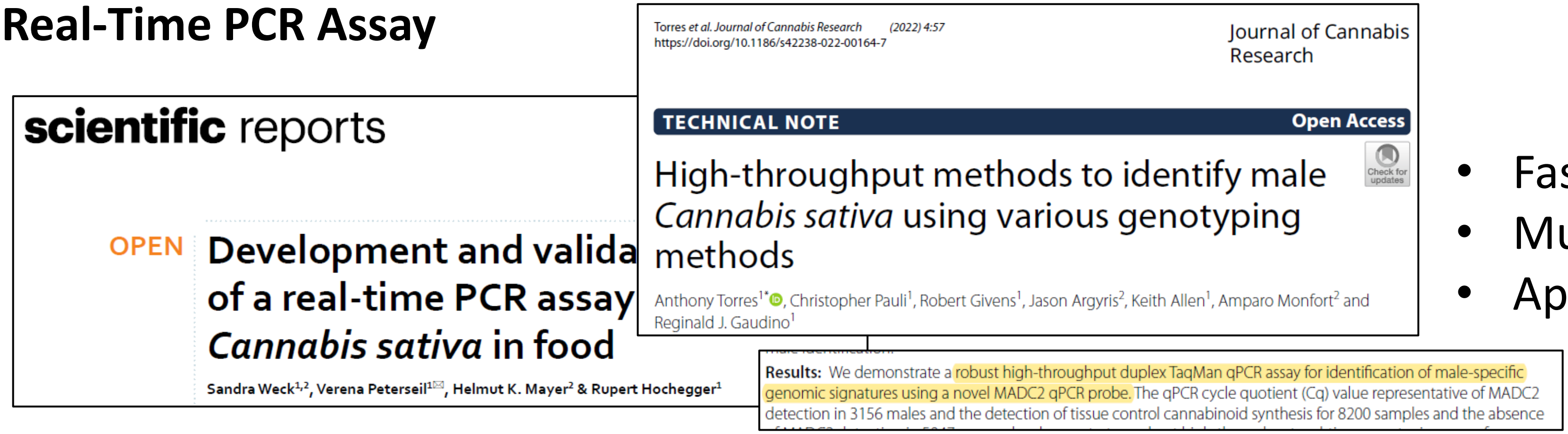
Geneious Prime software

Taqman qPCR Assay

Assays Evaluation

Development of TaqMan Probes qPCR Assay

➢ Real-Time PCR Assay



scientific reports

OPEN

Development and valida of a real-time PCR assay *Cannabis sativa* in food

Sandra Weck[1,2], Verena Peterseil[1✉], Helmut K. Mayer[2] & Rupert Hochegger[1]

Torres *et al. Journal of Cannabis Research* (2022) 4:57
https://doi.org/10.1186/s42238-022-00164-7

Journal of Cannabis Research

TECHNICAL NOTE Open Access

High-throughput methods to identify male *Cannabis sativa* using various genotyping methods

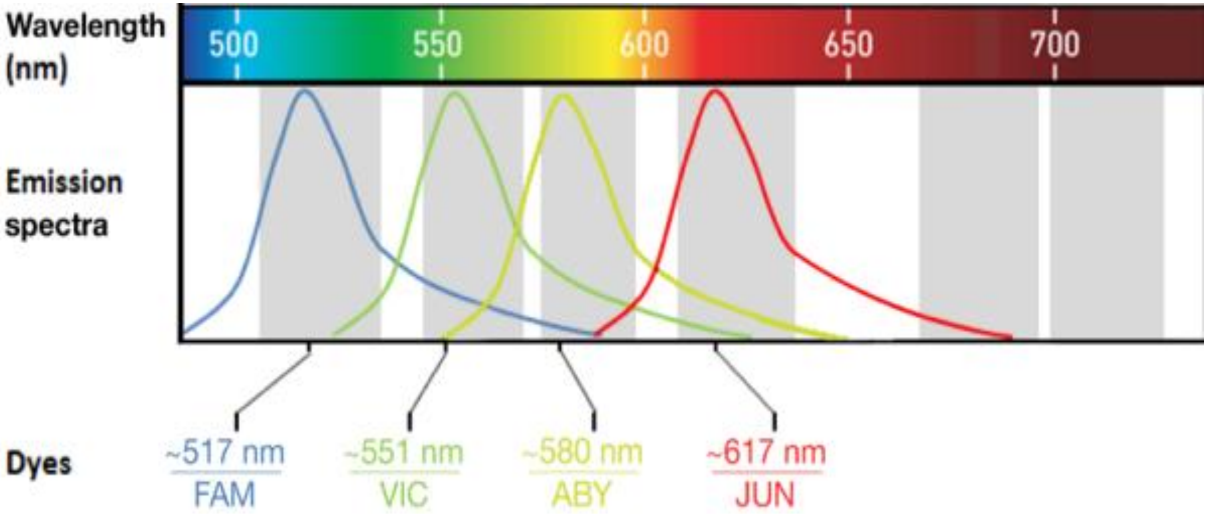
Anthony Torres[1*], Christopher Pauli[1], Robert Givens[1], Jason Argyris[2], Keith Allen[1], Amparo Monfort[2] and Reginald J. Gaudino[1]

Results: We demonstrate a robust high-throughput duplex TaqMan qPCR assay for identification of male-specific genomic signatures using a novel MADC2 qPCR probe. The qPCR cycle quotient (Cq) value representative of MADC2 detection in 3156 males and the detection of tissue control cannabinoid synthesis for 8200 samples and the absence

- Fast and easy to used
- Multiple targets
- Application-specific assay result

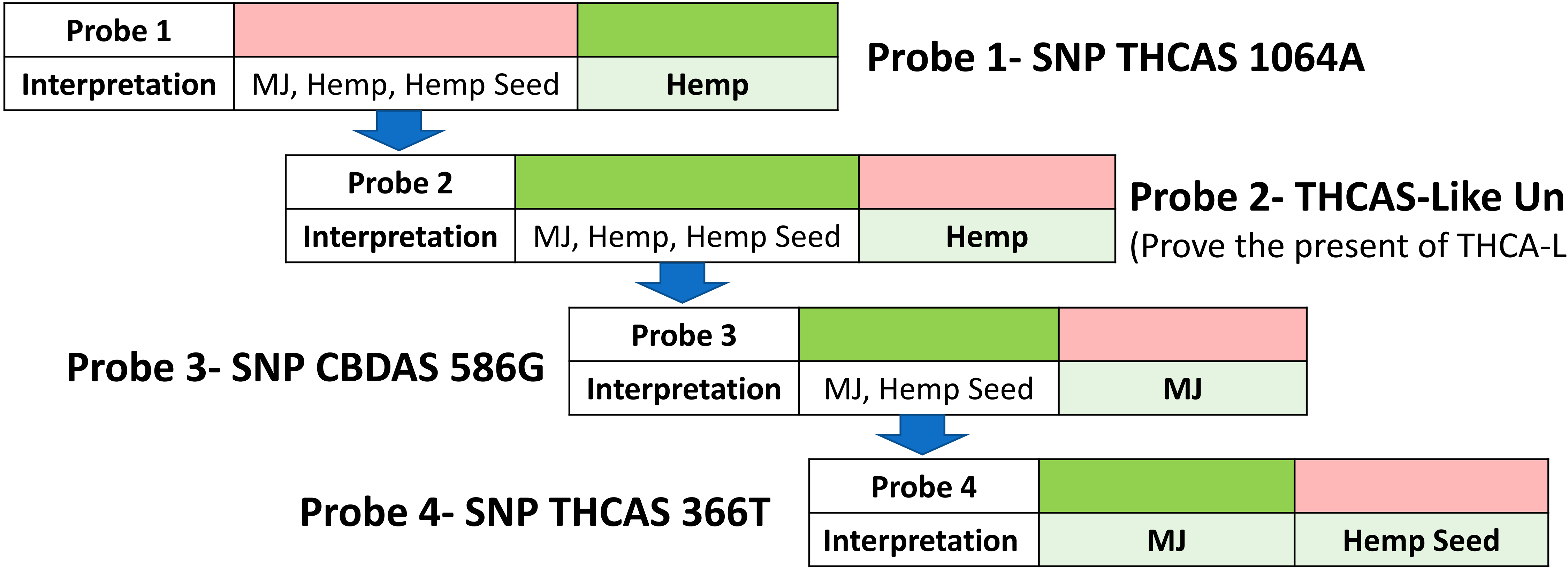
➢ Custom TaqMan Probes qPCR Assay

- Multiplexed detection of up to four targets (FAM, VIC, ABY, and JUN dyes)
- Designed 4 primer sets (eight primer, 50-150 bp amplicon)
- Four targeted probes with four dye colors
- Using TaqMan Multiplex Master Mix



TaqMan Probes qPCR Assay-Multiplex

- **For all Hemp, Hemp Seed, Seized MJ, Reference Sample (n=148)**



Probe 1	🟥	🟩
Interpretation	MJ, Hemp, Hemp Seed	**Hemp**

Probe 1- SNP THCAS 1064A

Probe 2	🟩	🟥
Interpretation	MJ, Hemp, Hemp Seed	**Hemp**

Probe 2- THCAS-Like Unique SNP
(Prove the present of THCA-Like gene)

Probe 3- SNP CBDAS 586G

Probe 3	🟩	🟥
Interpretation	MJ, Hemp Seed	**MJ**

Probe 4- SNP THCAS 366T

Probe 4	🟩	🟥
Interpretation	**MJ**	**Hemp Seed**

Differentiate hemp and marijuana (n=146) including 10 different sample types

Future Study-Assay Validation

Blind testing Cannabis sample to validate the application of new qPCR identification assay

Strain	Number	Source	Chemistry Data
US-Mexico Marijuana	60	CBP LSSD	Not Available
Chile Marijuana	20	PDI	Not Available
Chile Medical Marijuana	4	PDI	Not Available
Commercial Hemp	Need More!	Shops	Hopefully Available
Seized Marijuana	Need More!		Hopefully Avaliable
Reference Sample	Need More!		Hopefully Avaliable
Hemp Seed	Need More!	Shops	Hopefully Avaliable
Other Type of Sample	Needed		Hopefully Available
Total	150-200		

Summary

- Demonstrated the usage of the custom targeted NGS panel that serves as a comprehensive analysis of the three synthase genes and their pseudogenes to discover the relationship between chemotype and gene copies.
- With wider variety of sample types, hemp and marijuana cannot be differentiated look at only THCAS and CBDAS gene.
- Potential comprehensive workflow to differentiate cannabis crop types with not only synthase genes but the pseudogene.
- Developing a rapid genetic assay (qPCR) for the differentiation of hemp and marijuana (validation need in future study).

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- Center for Advance Research in Forensic Science (CARFS)
- National Institute on Drug Abuse (NIDA) – Drug Supply Program
- National Institute of Standards and Technology (NIST)
- University of Mississippi

CENTER FOR ADVANCED RESEARCH
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NIST
National Institute of
Standards and Technology

NIDA
NATIONAL INSTITUTE
ON DRUG ABUSE

MEMBER THE TEXAS STATE UNIVERSITY SYSTEM

THANK YOU FOR YOUR ATTENTION

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